Published in final edited form as:

Nanomedicine. 2014 July ; 10(5): 1013–1019. doi:10.1016/j.nano.2013.12.009.

Biomechanical Profile of Cancer Stem-like/Tumor Initiating Cells Derived from a Progressive Ovarian Cancer Model

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Abstract

We herein report, for the first time, the mechanical properties of ovarian cancer stem-like/tumor initiating cells (CSC/TICs). The represented model is a spontaneously transformed murine ovarian surface epithelial (MOSE) cell line that mimics the progression of ovarian cancer from early/non-tumorigenic to late/highly aggressive cancer stages. Elastic Modulus measurements via Atomic Force Microscopy (AFM) illustrate that the enriched CSC/TICs population (0.32 ± 0.12 kPa) are 46%, 61%, and 72% softer ($p < 0.0001$) than their aggressive late stage, intermediate, and non-malignant early stage cancer cells, respectively. Exposure to sphingosine, an anti-cancer agent, induced an increase in the elastic moduli of CSC/TICs by more than 46% (0.47 ± 0.14 kPa, $p < 0.0001$). Altogether, our data demonstrate that the elastic modulus profile of CSC/TICs is unique and responsive to anti-cancer treatment strategies that impact the cytoskeleton architecture of cells. These findings increase the chance for obtaining distinctive cell biomechanical profiles with the intent of providing a means for effective cancer detection and treatment control.

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Conflict of interests: none to disclose

This study provides insight into the biomechanical profile of Cancer Stem-like/Tumor Initiating cells (CSC/TICs) enriched from progressive stage of murine ovarian surface epithelial (MOSE) cancer cell line. Based on an established model for MOSE cells, on average, the cells exhibit an increasing deformability and biomechanical homogeneity as they transition to more aggressive phenotype. In this study, the results highlight the extremely deformable nature of CSCs/TICs in comparison to the elastic modulus values measured from MOSE cells in the progression of cancer. Furthermore, Stiffness profiling provides a suitable screening assay for drug treatment development and efficacy studies with compounds that specifically revert their deformability to a less aggressive phenotype.

Keywords

Cancer Stem-like/Tumor Initiating Cells; Cell Biomechanics; Atomic Force Microscope; Cancer Chemotherapy; Cellular Differentiation

Introduction

The research on stem cells has achieved great momentum in the recent decades. According to the traditional cancer model, often referred to as the clonal evolution model,¹ non-transformed cells can, in time, acquire an aggressive, dysregulated phenotype through the accumulation of multiple genetic or epigenetic alterations, ultimately leading to cancer. A key point in this assertion is that all cancer cells have the same potential for reinitiating a tumor. In other words, any cancer cell of a malignant tumor has the capability to form an identical tumor, if shed or re-implanted. Alternatively, a recently established cancer stem-like cell model suggests that only a unique and rare subset of cells within any given tumor are responsible for the growth, maintenance, and re-initiation of tumors.² It has been further suggested that the inability of today's therapeutics to target and eliminate those self-renewing "cancer stem-like cells" (CSCs) or "tumor initiating cells" (TICs) may be the cause for disease recurrence following chemotherapy; CSC/TICs are generally more resistant to chemotherapeutics compared to their fully differentiated tumor cell counterparts.^{3, 4} The current challenge is to determine the self-renewal capacity of individual tumor cells in order to assess the validity of either model in a specific tumor type.

For some time now, it has been claimed that the deformation characteristics of cells, as determined by their cytoskeleton organization may be a potential biomarker or predictor of cells' disease status.^{Suresh, 2007 #51} overcoming the substantial individual differences in the expression of oncogenes in individual tumors that hinder cancer detection. Our recent studies in a progressive murine ovarian cancer model^{5, 6} have demonstrated that cells exhibit an increasing deformability pattern and biomechanical homogeneity as they transition to more aggressive phenotypes;⁷ this is associated with changes in the actin cytoskeleton, with little to no effect by the microtubule network.⁸ Our reports are in agreement with results from other studies on breast,^{9, 10} prostate,¹¹ bladder,¹² melanoma,^{G. Weder, 2013 #1} and ovarian¹³ cancer cells, suggesting that cell stiffness is inversely related to tumorigenesis and metastatic potential.

Previously, studies on the stiffness of embryonic stem cells revealed that these cells are considerably softer before undergoing cellular differentiation process.¹⁴⁻¹⁶ Also, a recent study using a mechanical separation microfluidic chip has shown that breast TICs exhibit a more uniform deformability compared to more differentiated cancer cells.¹⁷ However, it has yet to be established whether CIC/TICs from other cancer types exhibit a similar deformability or whether anti-cancer compounds can modulate their deformability. Furthermore, the potential differences or similarities between the biomechanical properties of embryonic stem cells and CIC/TICs are yet to be identified.

In this study, we utilized the mouse ovarian surface epithelial (MOSE) cancer progression model, which has been shown to mimic many of the properties of benign and malignant

human ovarian cancer cells.^{5, 6, 18} Previously, we reported the increasing deformability of MOSE cells as they transition from early/benign to late/aggressive phenotypes.⁷ Here, we have extended these observations and evaluated the biomechanical properties of the CSC/TICs population using AFM nanoindentation. The CSC/TICs were enriched from three distinct aggressive variant cell lines that display different degrees of tumorigenicity as defined by their *in vivo* tumor growth rates. By determining their mechanical phenotype and classification, we assessed the degree to which the CSC/TICs conformed to the biomechanical profiling of our established MOSE cancer model.⁷

Additionally, we utilized our MOSE CSC/TIC population to assess the effect of serum-induced differentiation on the biomechanical signature of CSC/TICs. Cell differentiation is an important biological process, by which an unspecialized cell responds to environmental cues and develops into a more distinct cell type. A plethora of epigenetic and gene expression changes are associated with this process. Differentiation can dramatically change the cell size, shape, membrane potential, metabolic activity, and responsiveness to exogenous signals.¹⁹⁻²¹ Epithelial cancers are often associated with a de-differentiation phase, often referred to as epithelial-mesenchymal transition (EMT).²²

Finally, we investigated whether the sphingolipid metabolite, sphingosine (*So*), an anti-cancer agent that inhibits or delays cancer cell proliferation, is able to modulate the deformability of MOSE CSC/TICs towards a less aggressive signature.²³ Our results thus far have indicated that CSC/TICs exhibit distinct biomechanical properties that can be modified by anti-cancer treatment with the sphingosine.¹⁸

Methods

Hertz Model

Cell stiffness can be extracted by fitting the applied force-induced deformation data (force curves) with Sneddon's modified version of the Hertz contact model.²⁴ This model states that external forces are dominant over microscale surface and adhesive forces.^{24, 25} By asserting the applicability of classical mechanics theory in nanoscale indentation caused by the AFM tip, the Hertz model expresses the relation between an applied normal force by a spherical body and a sample's indentation:

$$F = \frac{4}{3} E^* \sqrt{R} \cdot \delta^{\frac{3}{2}} \quad (1)$$

Where F is the applied force, E^* is the relative stiffness term, R is the radius of the indenting body, and δ is the sample's induced deformation. In accordance with prior literature,^{26, 27} the equation is transformed into the following form:

$$F = \frac{4}{3} \sqrt{R} \frac{E_{cell}}{1 - \nu_{cell}^2} \cdot \delta^{\frac{3}{2}} \quad (2)$$

where E_{cell} and ν_{cell} are the elastic modulus and Poisson's ratio of the indented cell sample, respectively (cell assumed incompressible; $\nu_{cell}=0.5$). Furthermore, in the interest of

identifying the most accurate point of tip-sample contact, the indentation, δ , is both linearized^{28, 29} and referred to as the difference between the relative change in piezo position (z) and cantilever deflection (d):

$$\delta = (z - z_0) - (d - d_0) \quad (3)$$

Hence, the final form of the Hertz contact expression is as follows:

$$F^{\frac{2}{3}} = \left[\frac{4\sqrt{R}}{3(1-\nu_{cell}^2)} E_{cell} \right]^{\frac{2}{3}} (z - d) - \left[\frac{4\sqrt{R}}{3(1-\nu_{cell}^2)} E_{cell} \right]^{\frac{2}{3}} (z_0 - d_0) \quad (4)$$

Since the fitting model assumes linearity in the tip-sample interaction data, equation 4 can be analyzed such that the elastic modulus, E_{cell} , is determined from the slope, and the contact point (z_0, d_0) is approximated by the intercept of the line fit, respectively. Force curve analysis which includes the Hertz model fitting to the cell indentation data and calculation of cell elastic modulus, was performed with MATLAB software.

Atomic Force Microscope

Measurements were performed with the use of a Multimode V SPM integrated with a Nanoscope V controller (Veeco Instruments, Santa Barbara, CA). Uniform force loading was done at 1.5 ± 0.3 nN and a rate of $0.5 \mu\text{m/s}$ among all cell samples. Cell elastic moduli were measured from ~ 300 nm indentations in order to both maintain a constant convention and prevent deformations that exceeded 30% of total cell heights.^{30, 31} According to our SEM images, the total thickness of adherent MOSE cells is $1\text{--}2 \mu\text{m}$ for early stage cells while it is $3\text{--}4 \mu\text{m}$ for late stage cells. {A. Ketene, 2010 #1} Soft triangular-shaped cantilevers ($K \sim 0.02$ N/m, Olympus TR400PSA) were employed after modifying them with the addition of $\sim 10 \mu\text{m}$ diameter glass microspheres (Duke Scientific, Waltham MA); This promoted a better surface contact area between the probe and sample, enhanced the cell material homogeneity and isotropy approximation, and increased the likelihood that indentation was performed in the immediate nuclei region. Accurate measurements of glass microsphere diameters were made with a HIROX KH-7700 3D digital video microscope. All AFM tests were conducted within DMEM-HG cell culture medium at room temperature ($\sim 24^\circ\text{C}$) except designated *So*-treated cells which were tested within *So*-added DMEM-HG medium. For AFM experimentation, cells were grown and harvested within incubators at 37°C in humidified 5% CO_2 and later plated at 10^5 cells/slip on 0.15 mm thick, 12 mm² glass coverslips coated with 0.1 mg/mL collagen type IV (Sigma-Aldrich) for 24 hrs. $40 \mu\text{L}/3$ mL of HEPES at 1 M concentration was added to the cell samples to maintain a physiological pH of 7.2 during the experimentation. Final pH concentration of HEPES in the testing medium was 13.5 mM and stable for about two hrs, which was sufficient enough for each AFM test period.

Sample Preparation

MOSE cancer cells were generated and propagated as described previously.⁵ The cells are categorized into early (passage no. 15-25) (MOSE-E), intermediate (passage no. 60-90)

(MOSE I), and late stage (passage no. 110-200) (MOSE-L) based on their phenotype and genotype. Normal growth medium consisted of high glucose Dulbecco's modified Eagle's medium (DMEM-HG) supplemented with 4% Fetal Bovine Serum, FBS, 3.7g/L of sodium bicarbonate, and 1% Penn-strep solution.

The development of the Enhanced Green Fluorescent Protein-expressing (EGFP) and firefly luciferase-expressing (FFL) cell lines are reported elsewhere.^{32, 33} They both represent *in vivo* passaged variants derived from the MOSE-L cells; the MOSE-L^{EGFP} displays a moderate aggressiveness and results in slow-developing disease while the MOSE-L^{FFL} is highly aggressive and results in rapidly establishing tumors following intraperitoneal implantation in mice. For this study, MOSE CSC/TICs were enriched from the parental MOSE-L, MOSE-L^{EGFP} and MOSE-L^{FFL} variant cell lines by culturing under non-adherent spheroid culture conditions for isolation of cancer stem-like cells for other tumor types.^{34, 35} Briefly, cells were sequentially propagated under low attached conditions in serum-free DMEM:F12 (1:1) growth media supplemented with murine epidermal growth factor, (EGF; 20ng/ml), basic fibroblast growth factor (bFGF; 5ng/ml), insulin (5ug/ml) and bovine serum albumin (BSA; 0.2%). Of note, the majority (60-99%) of the cells initially die off over the first several passages resulting in the enrichment of CSC/TIC population. The enriched population that was able to exhibit clonal expansion from single cells is designated as a CSC/TICs population and has been stably maintained as spheroid cultures for at least 15 successive passages under these conditions.

To assess the impact of serum-induced differentiation on the biomechanics of CSC/TIC populations, single cells were seeded in the presence of complete DMEM-HG, supplemented with 4% FBS, for 24-48hrs prior to mechanical indentation experiments with the AFM.

For *So* modulatory therapy, CSC/TICs were cultured for at least three passages in the presence of exogenous *So*, added at a 1.5μM final concentration into complete DMEM-HG growth medium. This concentration was predetermined to be non-cytotoxic.

Statistical Analysis

Cells to be indented via AFM on the coverslips were arbitrarily selected, but had to macroscopically exhibit a well-adherent and healthy morphology/appearance prior to experimentation. The tested cells were only subjected to a single indentation, as repetitive indentations change cell morphology. {C. Zhu, 2000 #2} For indenting the cell nucleus region, the AFM tip was positioned at the center of cells. The experiments were conducted so as to acquire sample sizes of 40–50 cells for the previously reported regular MOSE cells and 75-105 for MOSE CSC/TICs from at least three separate AFM tests performed on different days. The Hertz model fit well with the acquired experimental data, with a high correlation coefficient ($0.85 < R^2 < 0.99$) to quantify the cells' elastic moduli. Statistical Shapiro-Wilks tests were employed to analyze the measured elastic moduli for normality distributions. Levene's statistical tests were used to test for homogeneity of variances. Two independent samples t-tests were also applied to the normalized data to evaluate the statistical significance of differences between the means of selected populations. A 95%

confidence interval ($p < 0.05$) was implemented to assess normality distribution of the data and the degrees of difference between two populations.

Results

A series of AFM nanoindentation tests were conducted on selected populations to measure cell stiffness (Figure 1). The elastic modulus parameter was measured on MOSE populations undergoing cancer progression representing early, intermediate, late stage cells (MOSE-E, -I, and -L), in addition to CSC/TICs enriched from the late stage cancer cell line variants. Moreover, the impact of re-differentiation as well as the effects of *So* treatment on CSC/TICs were investigated by comparing elastic modulus measurements following serum differentiation and *So*-treatment to that of untreated “control” cells.

The elastic modulus mean, standard deviation (SD), and mode values, (corresponding to the values most repeated in the sets of measurements) of the selected populations are represented in Table 1. Standard error of the means (SE) for the large selected sample sizes of the populations were very small, and were deemed negligible in terms of statistical significance. AFM measurements clearly show significant differences in stiffness of MOSE cells in three stages of cancer progression, confirming our previous results.⁷ The elastic modulus values showed that MOSE-L cells (0.60 ± 0.21 kPa) are on average 29% ($p < 0.0001$) softer than MOSE-I cells (0.83 ± 0.32 kPa) which are 27% ($p < 0.0001$) softer than MOSE-E cells (1.16 ± 0.56 kPa). Most notably however, the CSC/TICs population (0.32 ± 0.12 kPa) enriched from MOSE-L cells has shown to be 46% softer than MOSE-L cells ($p < 0.0001$), which provides additional insight into the biomechanical profiling scheme for the MOSE cancer progression model.

The CSC/TIC re-differentiation by growth in the presence of complete media containing serum did not elicit any significant change in the CSC/TICs’ mechanical response to external loading (0.33 ± 0.13 kPa). This suggests that short-term growth under differentiation conditions does not result in cell stiffness reversion. In contrast, *So* treatment caused a significant increase in the average elastic modulus value of the CSC/TICs (0.47 ± 0.14 kPa) by approximately 47% ($p < 0.0001$), indicating that MOSE CSC/TICs are biomechanically influenced by the treatment of the *So* anti-cancer agent (Table 1).

As depicted in Figure 2, Cell population histograms are generated by combining all recorded elastic modulus responses from each tested cell line. The data were best represented by log-normal distributions and log-Gaussian fits. The mode values may be extracted as the peak points of the histograms profile. The results from the Levene's tests rejected the equality of the histograms’ variances. Thus, it was concluded that there is a difference in the homogeneity of variances between the populations. Furthermore, the decrease in mean elastic modulus in cancer progression was accompanied by a decrease in standard deviation, and therefore variance of the histogram. This finding supports our previously established biomechanics model⁷ which indicates that cancer progression is associated with increasing deformability (Figure 2-A, 2-B, and 2-C), mechanical response homogeneity, and cytoskeleton disorganization. Most notably, the CSC/TIC population exhibited a more homogeneous stiffness profile and a very sharp concentrated histogram distributed in very

low elastic modulus values in comparison to other MOSE-derived cancer cells. MOSE CSC/TICs mechanical response distributions before and after differentiation and *So* treatment are shown in Figure 2-D, 2-E, and 2-F, respectively. Serum-induced differentiation had no impact on the histogram curve due to the short-term differentiation process (Figure 2-E) while *So* treatment notably shifted the response curve towards the “stiffer” values for CSC/TICs MOSE (Figure 2-F)

To confirm the unique, low stiffness response of CSC/TICs, the elastic modulus parameter for the CSC/TICs^{FFL} and CSC/TICs^{EGFP} populations were measured and compared to their parental counterparts. The AFM nanoindentation measurements are summarized in Table 2. The results illustrate that both CSC/TICs^{FFL} and CSC/TICs^{EGFP} populations display significantly (about 47% and 40%, respectively) less average stiffness compared to that of the corresponding parental MOSE-L cells (Table 2), and thus, confirm the distinctive CSC/TICs response profile. The distributions of the CSC/TICs populations are concentrated in lower stiffness values, and are more homogeneous than those of their respective parental MOSE-L variant cell lines. Column graphs representing mean values and error bars representing standard deviation of the measured elastic moduli for all selected populations are demonstrated in Figure 3. Therein, t-tests are applied to the logarithmic transformed and normalized data based on the Shapiro-Wilks test and the results from the t-tests (*p*-values) are illustrated and compared amongst each two populations.

Discussion

CSC/TIC populations enriched from three distinct, late stage variants of MOSE cancer cells exhibit a more homogeneous biomechanical signature in comparison to their parental counterparts. The CSC/TICs' stiffness distributions are in the very low elastic modulus values, which are a subset found in every transitional stage of this model including the early, intermediate, and late stages. Also, the short-term differentiation process did not cause any shift or change in the elastic modulus measurements. Finally, CSC/TICs, like their parental counterparts, are sensitive to *So* treatment, which targets cytoskeleton dysfunction in CSC/TIC phenotypes and reverts their deformability to a less aggressive phenotype.

The presented results have several implications. Most importantly, they may help explain some key questions about the nature of the CSC/TICs phenotypes. In particular, are CSC/TICs present throughout cancer progression or do they arise following accumulation of genetic alterations conferring the cancer-like phenotype? If the notion is followed that CSC/TICs do not develop with time, but are present at low levels, even at the earliest stages of cancer transformation, then softer cell types should be evident in all transition phases of our model, including the heterogeneous MOSE-E populations. These cells should be present with very high deformability profiles comparable to “cancer stem-like” or “tumor initiating” cells. However, it is unclear whether these early stage, relatively soft precursor cells would have acquired the necessary mutations or epigenetic changes to confer tumorigenicity but they may represent the earliest precursor tumor cell that is subject to further transformation. Hence, the deformability of cells may provide a signature to identify early premalignant cells. We are currently attempting to isolate the CSC/TIC populations derived from the MOSE-E (benign) and MOSE-I (intermediate stage) cell lines to assess whether

biomechanical differences are inherent to culture conditions or are clear indices for pre-malignancy or aggressive phenotypes. Of note, the enrichment for CSC/TICs resulted in cells that were either more or as aggressive *in vivo*, leading to higher tumor burden scores than their adherent counterparts, supporting the fact that they do in fact represent true tumor-initiating cell populations (Roberts, personal communication).

In agreement with our previous studies using adherent cultures of MOSE cells, MOSE CSC/TICs are susceptible to biomechanical modulation via exposure to non-cytotoxic doses of *So*. Based on our previous studies,⁸ the significant changes in cell stiffness most probably reflect changes in the cytoskeleton architecture of the CSC/TICs. *So* treatment over time improves cytoskeleton organization and increases f-actin levels in MOSE-L cells¹⁸ and increases the average elastic modulus of aggressive MOSE cells,³⁷ while not affecting their precursor benign cells. {Babahosseini H, 2013 #50}

The presented work suggests that MOSE CSC/TICs mechanical behavior conforms to the progression of our established MOSE cancer model⁷ and carries a unique distribution profile. Our study also validates the mechanical properties of CSCs derived from breast cancer cell lines¹⁷ and extends them to CSC/TICs derived from murine ovarian cancer cell lines. Future studies will clarify whether the softer biomechanical profile is a hallmark signature of all CSC/TICs irrespective of origin. The reported findings provide quantitatively supportive data that imply that the biomechanical properties of cells may be suitable for targeted isolation of malignant cells using biomedical microdevices. Indeed, the AFM technique may not prove to be amenable to high throughput cell identification or sorting based on the “deformability biomarker”, but other recent techniques such as the use of microfluidics³⁸⁻⁴⁰ could be employed for effective screening for stiffness and viscoelastic behavior of cells and as a means of biomechanical profiling of aggressive phenotypes.

Acknowledgments

We thank the staff of the Nanoscale Fabrication and Characterization Laboratory (NCFL) at Virginia Tech's Institute for Critical Technologies and Applied Sciences (ICTAS) for technical and instrumental supports.

Financial support: NSF ECCS-0925945 (to MA), NIH R01 CA118846 (to EMS and PCR), and the VT Fralin Research Institute Cancer Biology Initiative (to PCR and EMS).

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Figure 1.

AFM indentation top view screenshot during cell experimentation (Left) and indentation side view schematic illustration (right). AFM microcantilever is about 200 μm in length and the indenting glass sphere is about 10 μm in diameter. Cell indentations are usually in the sub-micrometer ranges as a result of applied nanoforce.

Figure 2.

Population distributions responses of (A) early, (B) intermediate, (C) late MOSE cells and the MOSE-CSC/TICs in (D) control (E) differentiated, and (F) *So* treated conditions.

Figure 3.

Elastic modulus responses of three successive stages of MOSE cancer cells and three variants of MOSE CSC/TICs enriched from late stage cell lines. In comparison to the established MOSE cancer stage model, the tumor initiating phenotype matches well in the order of cancer malignancy-mechanics functionality profile.

Table 1

Summary of elastic modulus responses for the early, intermediate, late MOSE cells and the control, differentiated, and So treated MOSE CSC/TICs.

MOSE Cells E_{elastic} (kPa)	Early Stage	Intermediate Stage	Late Stage	Control CSC/TICs	Differentiated CSC/TICs	So treated CSC/TICs
<i>Mean±SD</i>	1.164±0.557	0.825±0.324	0.601±0.212	0.321±0.119	0.333±0.125	0.471±0.138
<i>Mode</i>	0.95	0.7	0.5	0.25	0.28	0.42
<i>n Cells</i>	49	50	42	105	76	97

Table 2

Summary of elastic modulus values for the MOSE-L^{FFL} and MOSE-L^{EGFP} and the corresponding enriched CSC/TICs.

MOSE cells E _{elastic} (kPa)	MOSE-L ^{FFL}	MOSE-L ^{EGFP}	CSC/TICs ^{FFL}	CSC/TICs ^{EGFP}
<i>Mean±SD</i>	0.657±0.194	0.682±0.229	0.347±0.148	0.407±0.179
<i>Mode</i>	0.59	0.6	0.25	0.25
<i>n Cells</i>	55	44	48	49